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# Altered distribution of intraglomerular immune complexes in C3-deficient mice

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## SUMMARY

We have studied the role of complement in a model of glomerular inflammation induced by the *in situ* formation of immune complexes along the glomerular basement membrane. In C3-deficient mice, produced by homologous recombination, immune complex formation occurs initially in the subendothelial site and progresses slowly to the subepithelial position, whereas wild-type mice do not develop subendothelial deposits. In addition, the accumulation of electron-dense deposits is greater in the complement-deficient mice. Complement therefore influences glomerular handling of immune complexes, possibly because of changes in the physiochemical characteristics of the immune complexes. However, despite evidence of complement activation in the wild-type mice, as demonstrated by immunohistochemical detection of C3, C4 and C9, the degree of proteinuria was similar in C3-deficient mice. We conclude that, although complement is required for the normal glomerular metabolism of immune complexes, other, complement-independent, factors are involved in the generation of glomerular injury in this model.

## INTRODUCTION

Formation of immune complexes (ICs) within the glomerulus occurs in many immune-mediated renal diseases including immunoglobulin A (IgA) nephropathy, membranous nephropathy and systemic lupus erythematosus (SLE). The position of the IC within the glomerulus varies in different diseases and may relate to, and possibly determine, the type of injury that occurs. Despite this, the factors that determine the location of ICs are not fully understood. Indeed the mechanism by which ICs arise in the kidney, either *in situ* formation or by deposition of circulating complexes, is still a matter of debate.[1]

Animal models have been extensively used to study IC-mediated glomerular disease. One group of models relies upon the natural or chemically induced cationic charge on proteins to preferentially target them to the anionic glomerular basement membrane (GBM).[2,3] Once planted within the GBM, these proteins act as target antigens against which host antibody can bind, leading to *in situ* IC formation. This mechanism has been proposed to explain IC formation in human membranous nephropathy.[4,5] In support of this, the cationic protein-induced animal models have many features in common with human membranous nephropathy, in particular subepithelial IC formation, epithelial cell dysfunction, minor cellular infiltrate and proteinuria.

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The complement system is a series of soluble and membrane-bound proteins forming part of the body's innate immune system. It has many diverse immunological functions including cell lysis, opsonization, chemotaxis and modification of B- and T-cell responses.[6] The component C3 has a pivotal role in activation and a deficiency of this protein removes the majority of complement effector functions. Inappropriate or excessive activation of this system may contribute to tissue injury. Complement is present within the ICs of membranous nephropathy and has been proposed as a possible mediator linking antibody deposition with glomerular dysfunction and tissue injury.

Complement also has a role in the elimination of ICs.[7] The high density of antibody Fc regions within an IC will activate complement leading to C3 integration within the complex,[8] facilitating binding to complement receptor 1 on erythrocytes and IC removal by the reticuloendothelial system.[9] In addition, the presence of complement within a complex alters its size and structure, increasing solubility and solubilizing preformed tissue complexes.[10]

The handling of complexes within the glomerulus may also be dependent on complement. Antigenic material is cleared from the glomerulus more slowly in the absence of complement.[11,12] Fujigaki *et al.* demonstrated that the depletion of complement by cobra venom factor (CVF) treatment slowed the transit of antigen across the basement membrane.[13] A similar observation has been made in the translocation of antibody in passive Heymann nephritis.[14]

We have therefore studied the translocation of ICs across the GBM in C3 gene knockout mice. We report that the complete absence of both circulating and locally synthesized

C3 delays the development of subepithelial ICs, with subendothelial complexes forming in the absence of complement activation. However, the loss of glomerular permselectivity was not affected by the absence of complement activation. Although a deficiency of C3 results in an abnormality of IC handling, it has only a limited affect on the development of disease in this model of glomerulonephritis.

## MATERIALS AND METHODS

*Animals*

C57BL/6 mice and New Zealand white rabbits were purchased from B & K Universal, Hull, UK. C3-deficient mice were generated by homologous recombination as previously described.[15] No C3 could be detected in the plasma of homozygous deficient ($C3^{-/-}$) mice by enzyme-linked immunosorbent assay (ELISA) with a sensitivity of 10 ng/ml. They were maintained in a pathogen-free environment, but were otherwise phenotypically normal. All procedures were performed in accordance with government regulations.

*Disease protocol*

Six-week-old female C57BL/6 and $C3^{-/-}$ mice, weighing 17–19 g, were injected at day −21 with 200 μg of cationized bovine gamma-globulin (cBGG) in incomplete Freund's adjuvant subcutaneously. BGG (Cohn fraction II, ICN, Basingstoke, UK) was cationized as previously described.[16] At day 0 a test bleed was performed and anti-cBGG antibody titres were measured by ELISA.[17] The mice were injected on days 1–3 with 250 μg of cBGG in phosphate-buffered saline (PBS) into the tail vein. Either early morning urine samples (group 1) or 24-hr urine collections (group 2) were taken on days 0, 2, 4, 6 and 10. Groups of at least six mice were killed at days 2, 4, 6 and 10. Serum was taken for measurement of urea levels. The kidneys were harvested for histological analysis.

*Assessment of glomerular functional injury*

Urinary protein concentration was assayed using the Sigma Diagnostics Microprotein PR assay kit (Sigma, Poole, Dorset, UK). Urinary and serum albumin was measured by the bromocrescol green method.[18] Qualitative assessment of proteinuria was made by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) of 20 μg of urinary protein. Blood urea was measured using the Sigma diagnostics reagent which is based on the method of Talke & Schubert.[19]

*Histological analysis*

Formalin-fixed, wax-embedded tissue was stained with haematoxylin and eosin and periodic acid Schiff reagent. Immunohistochemistry was performed on 5-μm thick sections of frozen tissue. For the detection of C3 and C4, sections were incubated with monoclonal rat anti-mouse C3 or C4 (Connex, Martinsreid, Germany), followed by fluorescein-conjugated rabbit anti-rat immunoglobulin (Dako Ltd, High Wycombe, UK). C9 was detected using rabbit anti-rat C9 (from Prof. B. P. Morgan, University of Wales, Cardiff, UK), and mouse IgG by fluorescein-conjugated rabbit anti-mouse IgG (Dako Ltd). The cBGG was visualized within the tissue using indirect immunofluorescence with affinity-purified polyclonal rabbit anti-cBGG followed by fluorescein-conjugated goat anti-rabbit IgG (Stratech Scientific Ltd, Luton, UK).

*Electron microscopy*

Tissue was prefixed with 2·5% glutaraldehyde in PBS for 2 hr at 4°, washed and postfixed with osmium tetroxide for 90 min. Sections were stained with lead citrate and viewed on a transmission electron microscope (Hitachi H7000). The electron micrograph images were digitized with a Kodak megaplus 1·4 square pixel monochrome array camera. The basement membrane length and cross-sectional area of electron-dense deposits were measured using optimus software (Optimus corporation, Fort Collins, CO). All complexes along three or four glomerular loops were assessed from four (days 4 and 6) and six (day 10) mice and the density of complexes along the GBM were expressed as cross-sectional area of IC ($\mu m^2$) per unit length of GBM (μm).

*Statistical analysis*

Analysis of variance was used for statistical testing.

## RESULTS

Although complement deficiency can result in a reduced antibody response, in this study the dose of antigen used was large and administered in adjuvant and therefore anti-cBGG titres, measured 3 weeks post-immunization, was similar in both the wild-type and C3-deficient mice ($4{\cdot}88 \pm 1{\cdot}9$ and $5{\cdot}09 \pm 1{\cdot}97$ mg/ml anti-cBGG immunoglobulin, respectively, not significant).

### Renal function

Proteinuria was present in both wild-type and C3-deficient mice from day 4 onwards (Fig. 1a). There was no significant difference in 24 hr urinary albumin loss between the wild-type and C3-deficient mice throughout the course of the disease (Fig. 1b). There was no qualitative difference in urinary proteins as assessed by SDS–PAGE (data not shown). However, serum urea was significantly higher in the wild-type mice, suggesting some degree of functional protection in complement-deficient mice (Table 1).

### Histological analysis

As shown in Fig. 2, the microscopic appearance of the kidney on day 2 was normal. By day 4 there was thickening of the walls of the capillary loops and an increase in matrix within the mesangium. This appearance was similar in both groups of mice. At day 6, these changes persisted, and in addition vacuoles were present within the glomeruli, developing within the glomerular epithelial cell cytoplasm. By day 10 some glomeruli showed extensive disruption of normal structure. Glomerular hypercellularity was not noted during the development of this disease.

From day 4 onwards, casts were present within the tubules of wild-type and C3-deficient mice. They were associated with tubular dilatation and atrophy. By day 10 there was a mild interstitial infiltrate in both groups.

### Immunohistochemistry

BGG (Fig. 3a) was detected within the glomeruli of both groups of mice in a peri-capillary pattern from day 2 onwards.

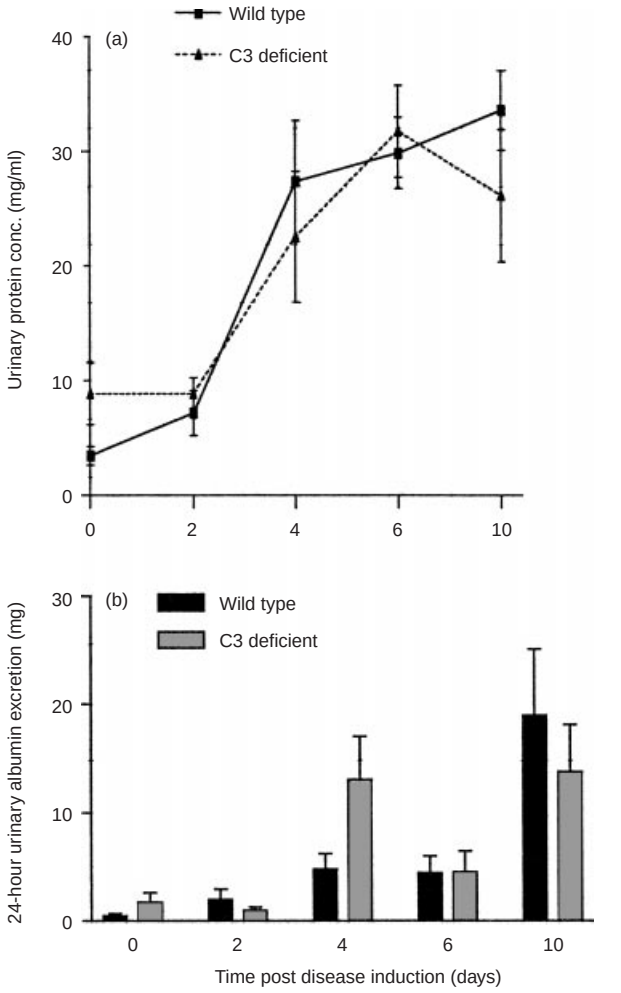


**Figure 1.** Proteinuria. (a) Both wild-type and C3-deficient mice became proteinuric 4 days after the induction of the disease (group 1, $n=6$–9 per group, mean $\pm$SEM). (b) There was no significant difference in 24-hr urinary albumin excretion at any time-point during the course of the disease (group 2, $n=9$ per group mean $\pm$SEM).

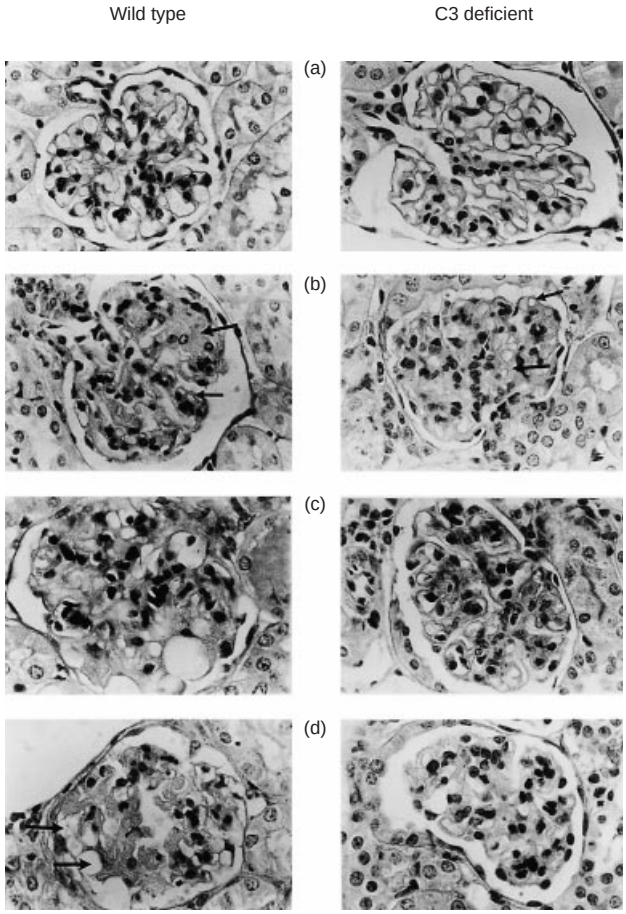


**Figure 2.** Histological assessment. Periodic acid Schiff staining ($\times 400$ original magnification) demonstrating progressive glomerular damage in wild-type and C3-deficient mice on days 2 (a), 4 (b), 6 (c) and 10 (d). There is evidence of capillary wall thickening (arrowed) and mesangial expansion (arrowed) from day 4 onwards. By day 6 the glomeruli contained large vacuoles (arrowed) with progressive disruption of glomerular morphology.

**Table 1.** Serum urea concentration in wild-type and C3-deficient mice

| | Wild-type | C3-deficient |
|---|---|---|
| Day 0 | 8·72 $\pm$ 0·57 | 7·80 $\pm$ 0·56 |
| Day 2 | 8·42 $\pm$ 0·76 | 8·30 $\pm$ 0·57 |
| Day 4 | 21·76 $\pm$ 6·67 | 29·56 $\pm$ 3·80 |
| Day 6 | 38·56 $\pm$ 11·02 | 21·92 $\pm$ 2·56 |
| Day 10 | 51·01 $\pm$ 4·38 | 22·93 $\pm$ 2·41 |

Data from the two experimental groups were combined. Wild-type and C3-deficient mice developed a significant rise in serum urea by day 4 (*$P<0·05$). By day 6 serum urea was significantly greater in the wild-type mice than the C3-deficient mice (†$P<0·05$). Serum urea measured in mmol/l, mean $\pm$SEM, $n>9$ per group.

Mouse IgG was seen in a similar distribution along the glomerular basement membranes, but was also present in the mesangium. This persisted throughout the course of the disease. No difference in intensity of immunoflourescence was detected between wild-type and C3-deficient mice. Complement staining demonstrated C3 within the glomeruli of the wild-type mice only (Fig. 3b) whereas C4 was present in both groups of mice. C9 was present within the glomeruli of wild-type mice, suggesting formation of the membrane attack complex.

### Electron microscopy

The deposition of ICs in wild-type mice differed in both position and quantity when compared with the C3-deficient mice. Electron-dense deposits were exclusively subepithelial in wild-type mice (Fig. 4) whereas in the C3-deficient mice the location was initially subendothelial, reaching a subepithelial site later in the disease (Table 2), although intramembranous deposits persisted to day 10 (Fig. 5b). Secondly, by morphometric analysis, the density of ICs was significantly higher in the C3-deficient mice (Table 3) with almost contiguous stretches of IC along the GBM (Fig. 5c). The greater density of immune material in the C3-deficient mice did not, however, correlate with greater urinary protein loss.

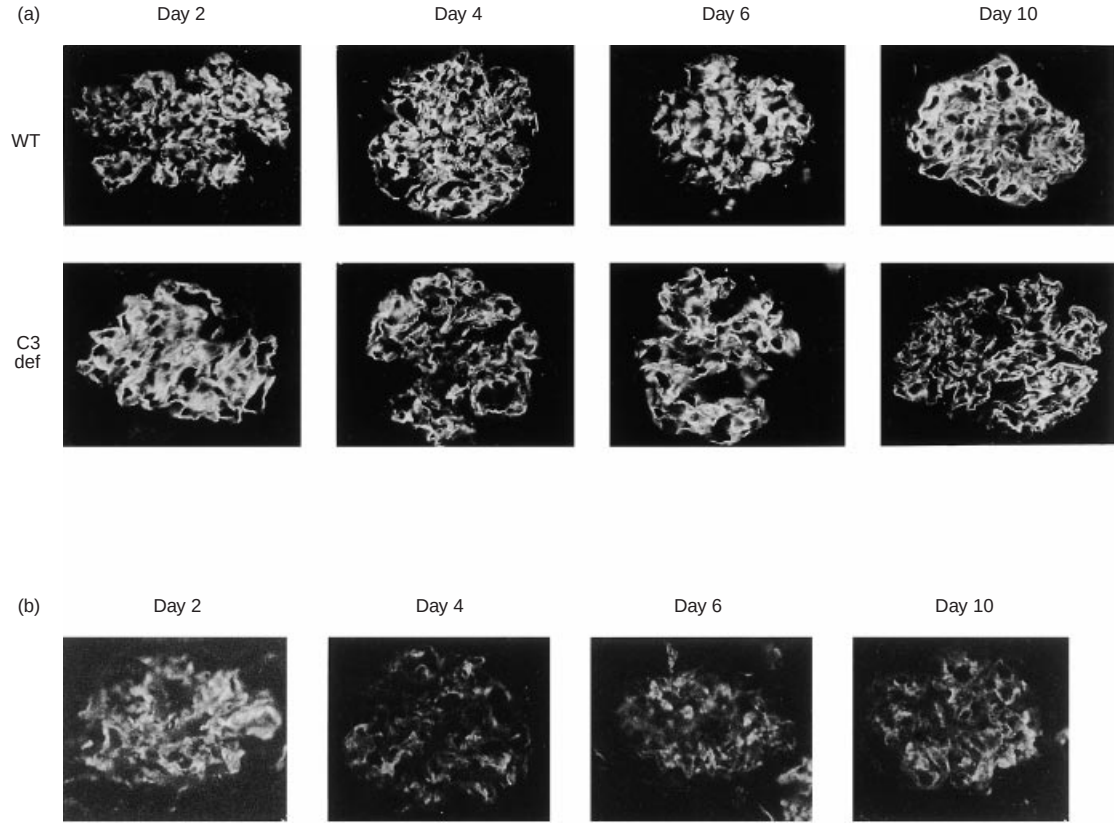


**Figure 3.** Immunochemical analysis of immunoreactants. (a) shows the distribution of cBGG at days 2, 4, 6 and 10 in both the wild-type and C3-deficient mice (original magnification ×400). A similar distribution and intensity of staining is seen in both groups of animals. (b) shows the distribution of C3 staining in the wild-type mice at the above time points. C3 staining was absent in the C3-deficient mice.

## DISCUSSION

We have demonstrated that a deficiency in complement activation due to the absence of C3 influences the distribution of ICs within the GBM and leads to an accumulation of electron-dense material in this model of glomerulonephritis. When CVF-depleted mice were used to study the distribution of ICs in cBGG nephritis, complexes were only formed in the subendothelial site during CVF treatment.[20] Our results suggest that a total deficiency in C3 appears to retard, but not inhibit, the passage of ICs across the GBM, with the subepithelial site of IC deposition predominating by day 10 in both groups. This effect on IC distribution may be more pronounced when, as is likely in human disease, there is an ongoing production of ICs.

Complement is likely to influence IC transition across the GBM by altering the characteristics of the IC. This mechanism may operate in physiological as well as pathological conditions since complement can be detected within the normal glomerulus.[21] C3 split products can impair Fc–Fc and interactions and disrupt the preformed antibody–antigen lattice, therefore preventing tissue deposition and solubilizing complexes. Fujigaki *et al.*,[13] using immunogold electron microscopy, demonstrated retardation of antigen transfer across the GBM in CVF-depleted rats. C3 staining colocalized with antigen in the subendothelial and subepithelial sites but was not seen within the GBM itself. This would suggest that complement induces a reversible disruption of IC structure, permitting transmembrane passage with subsequent re-assembly in the subepithelial position. It also raises the question as to the source of the complement during subepithelial complex re-assembly and the possibility of a contribution from epithelial cell synthesis.[22–24]

The quantity of electron-dense material in the glomeruli of the C3-deficient mice is greater than in those of wild-type mice. This could reflect increased production of complexes or reduced systemic or local clearance of ICs. As the anti-cBGG titres were similar immediately prior to the induction of the disease in the wild-type and C3-deficient groups the rate of production of ICs is likely to be similar. In addition, this disease is thought to be mediated via *in situ* IC formation rather than deposition of circulating complexes and therefore the influence of systemic IC formation and removal is likely to be small. We conclude therefore that a difference in the rate of removal of ICs from the glomerulus between the wild-type and C3-deficient mice is likely to exist. In addition, despite the increased accumulation of ICs in the glomeruli of the C3-deficient mice the degree of functional disturbance is not increased. This would suggest that the ICs do not alter function due to a simple disturbance of glomerular structure. The relationship between IC deposition and functional injury obviously depends on other aspects of the interaction between the complexes and the glomerular microenvironment other than the quantity of IC deposited.

The cBGG model of glomerular disease mimics human

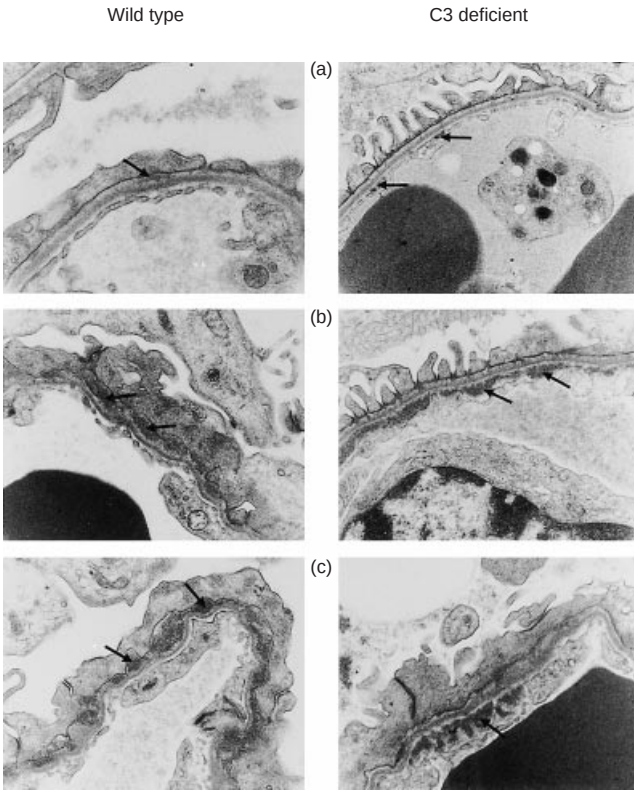


**Figure 4.** Electron micrographs (×20 000) at day 2 (a), 4 (b) and 6 (c). Electron-dense deposits (arrows) are in a subepithelial position in wild-type mice at all time-points. C3-deficient mice initially show subendothelial deposits with transition to a subepithelial site as the disease progresses. Epithelial cell foot process effacement is evident from day 4 onwards.

**Table 2.** The site of IC deposition assessed by visual examination of the electron micrographs

| | Wild-type mice | | C3-deficient mice | |
|---|---|---|---|---|
| | Sub-endo.* | Sub-epi. | Sub-endo. | Sub-epi. |
| Day 2 | – † | + + + | + + + | – |
| Day 4 | – | + + + | + + | + |
| Day 6 | – | + + + | + | + + |
| Day 10 | – | + + + | + | + + |

*Sub-endo., sub-endothelial; sub-epi., sub-epithelial.

† + + + exclusive site; + + predominant site; + minor site; or – absence of electron-dense deposits.

membranous nephropathy, insofar as an extrinsic antigen is planted within the GBM, against which an immune response is generated with the formation of ICs. The finding, in both human and experimental diseases, that complement components colocalize with the immune deposits has led to the hypothesis that complement activation represents a link between IC deposition and functional injury. However, the loss of glomerular permselectivity in this model, under the conditions described, is not dependent on complement activation.

In previous reports of the cBGG model of glomerulonephritis, CVF depletion greatly reduces the degree of func-

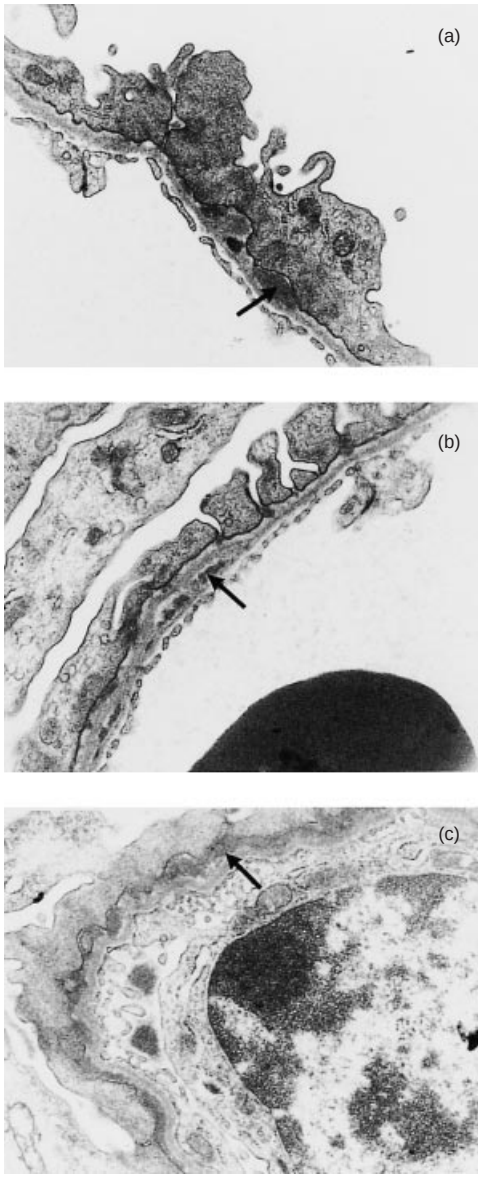


**Figure 5.** Electron micrographs (×20 000) at day 10. Wild-type mice have discrete subepithelial deposits (a) whereas C3-deficient mice have intramembranous deposits (b) and have lengths of basement membrane with an almost continuous band of electron-dense material (c) which are not seen in the wild-type mice.

**Table 3.** Quantification of IC deposition

| | Wild-type mice | C3-deficient mice | *P*-value |
|---|---|---|---|
| Day 4 | 0·0811 ± 0·143 | 0·141 ± 0·095 | 0·384 |
| Day 6 | 0·0475 ± 0·011 | 0·0848 ± 0·013 | 0·092 |
| Day 10 | 0·0542 ± 0·010 | 0·0901 ± 0·011 | 0·031 |

The density of ICs is greatest at day 4 in both groups. The subsequent decline in density of complexes is greater in the wild-type mice and by day 10 there is a significantly higher density of immune complexes in the glomeruli of the C3-deficient mice. Area expressed in $\mu m^2$ $\mu m$ of glomerular basement membrane.

tional injury, preventing the development of proteinuria.[20] In the present study, however, the C3-deficient mice develop proteinuria at the same stage and to the same extent as the wild-type mice. This may be related to the use of CVF to deplete complement in previous studies. CVF treatment results in the production of multiple immuno-modulatory peptides which may independently affect the pathophysiology of immune complex-mediated disease. The use of C3-deficient mice therefore provides a more definite account of the role of complement.

Alternatively, strain differences in the mice used may have affected disease expression, a phenomenon recognized in other disease models. The original report of cBGG-induced nephritis used BALB/c mice (H2d). In the nephrotoxic nephritis model of glomerulonephritis, BALB/c mice have higher levels of antibody production and more glomerular complement deposition than C57BL/6 mice (H2b).[25] In contrast C57BL/6 mice show a more cell-mediated pattern of injury. This tendency of C57BL/6 mice to mount a cell-mediated response may explain why complement deficiency does not provide protection from disease in the cBGG model and explain the difference from the original description.

As complement activation is not responsible for the loss of glomerular permselectivity an alternative mechanism must be involved. Since C57BL/6 mice mount such a T helper type 1 (Th1)-weighted response it is likely that cell-mediated injury is responsible for the disruption of function. There are parallels in other antibody-mediated disease models, in which, although antibody is required to target the glomerulus, T cells and macrophages are essential for the progression of the injury.[26]

The wild-type mice develop a significantly greater rise in serum urea concentration. This may be related to worse glomerular injury and loss of filtration in wild-type mice. Alternatively complement may mediate tubular and interstitial injury in the presence of proteinuria,[27,28] from which the C3-deficient mice are protected.

The glomerulus with its high blood flow and structural adaptation to filtering is continually exposed to antigen and IC trapping. It is therefore essential that a mechanism for removal of complexes exists. Disease could result if there is excessive systemic production or failure of local removal of ICs. In the context of our present understanding of this model, our study supports a role for complement in the local removal of complexes from the glomerulus. However, it demonstrates a limited role for complement in the generation of glomerular injury in this model.

## ACKNOWLEDGMENTS

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